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Genetic Assessment of Moroccan Tomato (*Solanum lycopersicum* L.) Genotypes by RAPD and SSR Markers

Amraoui Rajae[1], Dominique Mingeot[2], Mohamed Addi[1], Ahmed Elamrani[1], Hana Caid Serghini[1], Aatika Mihamou[1], and Malika Abid[1*]

[1] Laboratory of Biology of Plants and Microorganisms, Faculté des Sciences Oujda – Université Mohammed Premier, BP-717 Oujda, Morocco; [2] Laboratory of Wallonia Center of Agronomics Researchs (CRA-W), Département Sciences du Vivant, Bâtiment Jean-Baptiste de La Quintinie, Chaussée de Charleroi, 234 B-5030 Gembloux, Belgium

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Abstract

For the first time eight local tomato cultivars collected from four different regions of Morocco were assessed with RAPD and SSR methods. Most of RAPD markers give monomorphic banding profiles. Only OPU03 marker showed a total of 4 polymorphic amplicons out of 8 recorded in FIGUIG2 cultivar. The analysis with SSR markers gives more polymorphism. The number of alleles amplified assessed from 2 to 5 alleles among cultivars. The similarity matrix subjected by the unweighted pairgroup arithmetic method (UPGMA) clustering grouped the cultivars in four groups where FIGUIG2 cultivar formed a separate and more distant cluster. In addition this cultivar holds the very high percentage of uniformity (99%) indicating that is an homogeneous traditional cultivar with high purity. This genotype can be conserved and used in breeding programs. More traditional Moroccan cultivars must be collected in order to determine their genetic structure.

Keywords: Moroccan tomato cultivars, RAPD and SSR markers, genetic diversity.

Introduction

Tomato belongs to Solanaceae family and it is originated from the Andean region, more exactly from Ecuador, Bolivia, Colombia and Chile. Tomato seeds were introduced by Spaniards from Mexico to Europe for domestication (Peralta et al. 2006) and it was then referred as a cultivated plant in Italy. At the end of the XIXth century, tomato cultivars were self-pollinated and farmer saved seeds from a year to the other. New genotypes resulted from spontaneous mutations, natural outcrossing or recombination of pre-existing genetic variation (Bauchet and Causse, 2012). Commercial itinerary has contributed to spread the species worldwide (Diez and Nuez, 2008). This has led to collect existing genetic diversity to preserve and to valorise it all over the world through public or private institutes of plant germplasm.

The seed sector in Morocco has known since the early 70s, development and sustained growth that enabled the creation of a national plant genetic quality. However, tomato has not yet benefited from this progress and we do not yet have a certified Moroccan tomato cultivar.

In Morocco, the most tomato is grown in greenhouses under controlled climate. Nevertheless, almost all the seeds were bought from foreigner countries. The use of certified seed by breeders is the means to exploit and disseminate the most effective advances in seed breeding.

Characterization of tomato germplasm is of great impor-

* Corresponding author: abidmalika@yahoo.fr

tance for current and future agronomic and genetic improvement of the crop. Furthermore, if an improvement programme is to be carried out evaluation is imperative, in order to understand the genetic background and the breeding value of the available tomatoes.

Morphological, biochemical and molecular characterization are used to specify genetic diversity between tomato varieties (Garcia et al., 2004). Morphological and biochemical characterization is used to evaluate many fruit quality traits such as diameter, height, shelf life, weight size, acidity, colour and firmness. Those parameters do not always allow the quantification of genetic diversity in plants and are dependent of environmental factors (Cooke, 1994). Molecular markers are an efficient tool to investigate the genetic basis of agronomic traits and to make easier the transfer and accumulation of desirable traits between breeding lines. Many molecular techniques including amplified fragment length polymorphism (AFLP), restricted fragment length polymorphism (RFLP), simple sequence repeats (SSR) and random amplified polymorphic DNA (RAPD) were used to set up genetic variation in tomato cultivar collections (Bredemeijer et al., 1998; Park et al., 2004; Garcia-Martinez et al., 2006).

Local tomato germplasm may provide some natural variation that is present in the species as a whole (Brush, 2000; Feuillet et al., 2008) since local varieties represent the main source of genetic variation in the cultivated species. They can offer big interest for their use in scientific studies and in breeding programs (Chable et al., 2009). For this reason, eight local tomato genotypes and one French commercial tomato used as control were assessed using RAPDs and SSRs markers to examine the genetic variability, to establish their relationships and to compare the usefulness of these markers.

Materials and methods

Plant Materials

Nine lots of tomato were studied in this investigation including one commercial variety of French origin "Saint Pierre" from Vita Company used as a control. The other lots were collected from four different regions of Morocco and are listed as follow: From Berkane region (Northeast) two tomato lots noted BERKANE1 and BERKANE2, from Figuig region (South east), two tomato lots noted FIGUIG1 and FIGUIG2, from Rissani region (South west) three batches noted RISSANIB, RISSANIO and RISSANIN in which we found a difference in color between the seeds and finaly, one tomato lot from HOCEIMA region (Northern Morocco).

The seeds of each lot of tomato were sown in the greenhouse for germination and growth. After two weeks of sowing, the seedlings were transplanted and grown in green house at the nursery of the park Lala Aïcha with a regular watering.

Genomic DNA Isolation

For total genomic DNA extraction, fresh leave of each lot of tomato were grounded in liquid nitrogen and stored at -80°C. Genomic DNA isolation was performed following the procedure of the DNeasy Plant mini kit de Qiagen.

RAPD and SSR Primers

Ten RAPD random primers and fourteen microsatellite markers were used for molecular characterization of Moroccan tomato lots. All RAPD and SSR primers were chosen among the highly polymorphic primers published on the literatures (Suliman-Pollatschek et al., 2002; Areshchenkova and Ganal, 2002) and have been applied successfully for assessing different plant genotypes. The used RAPD primers are OPC09, OPU03, OPA14, OPU14, OPA15, OPB17, OPB18, OPC08, OPG17 and OPV19. The simple sequences repeat (SSR) primers are listed in Table 2.

RAPD-PCR Amplification

PCR assays was performed in a 25µl final volume, containing 20ng of genomic DNA, 0.2µM of operon random primer, 100µM dNTPs, 2.5mM MgCl2, 1mg /ml BSA, 5 X PCR reaction buffer, and 1 U Taq DNA polymerase (Promega).

The amplifcations were conducted with Thermal Cycler (Applied system), with an initial 5 min at 94°C that was followed by 45 cycles of 1 min at 94°C, 1 min 30 s at 36°C, and 2 min 30 s at 72°C, ended by 7 min extension at 72°C. PCR product were electrophoresed on 1.4% agarose gel stained with ethidium bromide and observed under UV light and photographed. Size of the amplicons was estimated with 1kb DNA ladder which was resolved along with amplified product. Reproducibility of the results was confirmed by repeating the amplification twice.

SSR-PCR Amplification

For SSR analysis, among the relatively high number of SSR loci already reported in tomato, 14 SSR markers were selected from the published data (Suliman-Pollatschek et al., 2002; He et al., 2003) or on the website of Solanaceae Genomics Network (http://solgenomics.net).

PCR amplification was performed in a 20 µl total volume, containing 20 ng of genomic DNA, 0.25 mM of each primer, 200 µM dNTPs, 1.5 mM MgCl2, 1 mg /ml BSA, 1 X PCR buffer, and 1 U Taq DNA polymerase (Promega).

The amplifications were conducted with Thermal Cycler (Applied system), with an initial 5 min at 94°C that was followed by 35 cycles of 30 s at 94°C, 45 s at X°C, and 1min 30 s at 72°C, ended by 7 min final extension cycle at 72°C. The amplification products were separated and analyzed on a Licor sequencer type (Westburg) using a 6.5% acrylamide gel. The lengh of the alleles was determined by comparaison with marker loaded on adjacent gel traks. The raw data were collected and analyzed by the analysis software "Gene ImageIR" (Westburg).

Cluster Analysis

All 9 varieties were clustered based on the estimated genetic distance. The positions of a consistent RAPD or SSR bands were scored and transformed into a binary character matrix "1" for the presence and "0" for the absence of a RAPD and SSR band at a particular position. Genetic similarities between genotypes

were calculated according to Nei and Li's Coefficient (1979).

The similarity matrix was subjected to cluster analysis by the unweighted pairgroup arithmetic method (UPGMA; Sneath and Sokal 1973) and phylogenetic tree was created using the output data and the graphical module of the MVSP 3.1 software.

Results and Discussion

For the first time in Morocco we studied the genetic variability of some local tomato cultivars collected from four different regions. Nine lots of tomato were selected in this study including one commercial French variety "Saint Pierre" obtained by Vita Company used as a control. The other tomato lots were collected from farmers and were sourced from different geographic regions which differ greatly in their agro-ecological and ethnic compositions. The farmers were asked specific questions as the local names for each tomato lot and the location where they are cultivated. Tomato genotypes collected from Berkane (Northeast) and Hoceima (North) regions are adapted to semi-dry climate but humid. Tomato genotypes from Rissani and Figuig regions are cultivated in a very dry environment. Tomato seeds from these regions are collected from a year to the other to maintain germplasm. These lots are usually grown in small fields and reserved to self-consumption or to local markets.

RAPD Markers

RAPD markers have great potential to evaluate genetic diversity within accessions and can provide much informations useful in breeding programs. RAPD analysis is technically easy, simple and can generate polymorphic profile suitable for large scale germplasm characterization (Rafalski and Tingey, 1993).

In our study, 10 RAPD markers were chosen for the analysis of tomato cultivars variation from four different regions of Morocco in a mixture of 5 plants per lot. The RAPD fragments were scored for their presence (1) and absence (0) for each sample and genetic diversity among groups was calculated on the basis of Nei & Li's Coefficient (1979). Out of these 10 primers, two primers (OPG17 and OPG19) did not achieve any molecular polymorphism. The same result was obtained with OPG17 primer in 19 Azerbaijan Tomato genotypes (Sharifova et al., 2013). The Rest of the primers have amplified a total of 41 signals out of which 5 were polymorphic and 36 monomorphic. The number of signals amplified by these markers varied between 2 for OPC08 marker and 8 for OPU03. OPC08 marker gives monomorphic banding patterns among all the cultivated cultivars (Figure1). Only random primer OPU03 showed a total of 4 polymorphic amplicons out of 8 amplicons recorded in FIGUIG2. In other study this percentage was much higher. For example, the same primer OPU03 produced highest number of polymorphic bands (21 bands) in 19 tomato varieties (Thamir et al. 2014). With this primer (OPU03), only cultivated tomato FIGUIG2 and BERKANE2 displayed a polymorphism showing 4 specific alleles for FIGUIG2 and only one allele for BERKANE1. This marker discriminate cultivars FIGUIG1, RISSANIB, BERKANE1 and HOCEIMA who have the same profile from RISSANIN, and RISSANIO who share the same profile with the control cultivar Saint Pierre indicating that they could have common origin. These results indicated the existence of limited genetic variation within the studied tomato Moroccan lots. Many others markers showed highly monomorphic profile in the cultivated *S. lycopersicum* L. (Labate and Roberts, 2002).

The values of pair-wise genetic distances ranged between 0,947 and 1 indicating low diversity in the studied tomato genotypes (Table 2). The highest genetic distance (1) was observed between some cultivars like HOCEIMA and FIGUIG1 whereas the lowest genetic distance (0,947) was detected between BERKANE2 and FIGUIG2 which is an evidence for a low genetic similarity value in the tomato germplasm studied. Low degree of the genetic variability is often correlated to a weak discrimination of RAPD markers. Moreover, Miller and Tanksley (1990) estimated that only 5% of genetic variation exist within *S. Lycopersicum*.

Nevertheless, these primers could dived Moroccan tomato varieties into four groups: the first includes FIGUIG1, RISSANIB, BERKANE1 and HOCEIMA lots. The second includes RISSANIN, Saint Pierre and RISSANIO. The third includes only BERKANE2

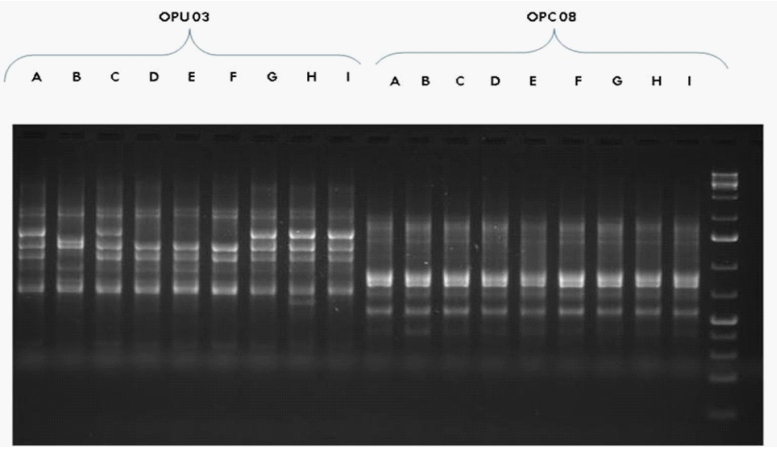

Figure 1. RAPD electrophoretic pattern of tomato cultivars obtained by the primers OPU 03 and OPC 08 (from left to right) (A: FIGUIG1 B: FIGUIG2, C: RISSANIB, D: RISSANIN, E: Saint Pierre, F: RISSANIO, G: BERKANE1, H: Berkane2, I: HOCEIMA). Right extreme lane represents 1kb DNA.

Table 1. Pair-wise genetic distances from 10 RAPD markers of eight tomato cultivars and one French commercial cultivar Saint Pierre.

	FIGUIG1	FIGUIG2	RISSANIB	RISSANIN	RISSANIO	BERKANE1	BERKANE2	HOCEIMA	Saint Pierre
FIGUIG1	1								
FIGUIG2	0,96	1							
RISSANIB	1	0,96	1						
RISSANIN	0,987	0,973	0,987	1					
RISSANIO	0,987	0,973	0,987	1	1				
BERKANE1	0,987	0,974	0,987	0,974	0,974	1			
BERKANE2	0,987	0,947	0,987	0,974	0,974	0,974	1		
HOCEIMA	1	0,96	1	0,987	0,987	0,987	0,987	1	
Saint Pierre	0,987	0,973	0,987	1	1	0,974	0,974	0,987	1

Table 2. Set of simple sequence repeats (SSR) primers used in this investigation.

Marker	Référence	Motif	Forward	Reverse	Chromosome	T° Hybridation
SSR14	http://solgenomics.net/	(ATA)9	TCTGCATCTGGTGAAGCAAG	CTGGATTGCCTGGTTGATTT	3	55°
SSR22	http://solgenomics.net/	(AT)11	GATCGGCAGTAGGTGCTCTC	CAAGAAACACCCATATCCGC	3	50°
SSR26	http://solgenomics.net/	(CGG)7	CGCCTATCGATACCACCACT	ATTGATCCGTTTGGTTCTGC	2	50°
SSR63	http://solgenomics.net/	(AT)39	CCACAAACAATTCCATCTCA	GCTTCCGCCATACTGATACG	8	55°C
SSR248	http://solgenomics.net/	(TA)21	GCATTCGCTGTAGCTCGTTT	GGGAGCTTCATCATAGTAACG	10	55°
SSR578	http://solgenomics.net/	(AAC)6(ATC)5	ATTCCCAGCACAACCAGACT	GTTGGTGGATGAAATTTGTG	6	55°
Tom236-237	Suliman-Pollatschek *et al.* 2002	AT16	GTTTTTTCAACATCAAAGAGCT	GGATAGGTTTCGTTAGTGAACT	9	47°C
TOM184	Suliman-Pollatschek *et al.* 2002	(ATTT)3 (ATT)7	CAACCCCTCTCCTATTCT	CTGCTTTGTCGAGTTTGAA	4	45
TOM196-197	Suliman-Pollatschek *et al.* 2002	(GA)14	CCTCCAAATCCCAAAACTCT	TGTTTCATCCACTATCACGA	11	45
TOM210-211	Suliman-Pollatschek *et al.* 2002	(ATA)15	CGTTGGATTACTGAGAGGTTTA	ACAAAAATTCACCCACATCG	4	45
TMS52	T. Areshchenkova · M.W. Ganal. 2002	(AC)14 (AT)18	TTCTATCTCATTTGGCTTCTTC	TTACCTTGAGAATGGCCTTG	12	55
TMS56	T. Areshchenkova · M.W. Ganal. 2003	(CT)19	GATCTCAAAGGATGAACAATAC	TCATTAGGAGATTCTTTGTATCA	1	55
TMS63	Areshchenkova and Ganal 2002	(AT)4(GT)18(AT)9	GCAGGTACGCACGCATATAT	GCTCCGTCAGGAATTCTCTC	1	60°C
TMS65	T. Areshchenkova · M.W. Ganal. 2002	(TA)25 (GA)20	AGCTTCATCCATTACGCCAC	GTGCATCTGGCGTACCTACC	12	60

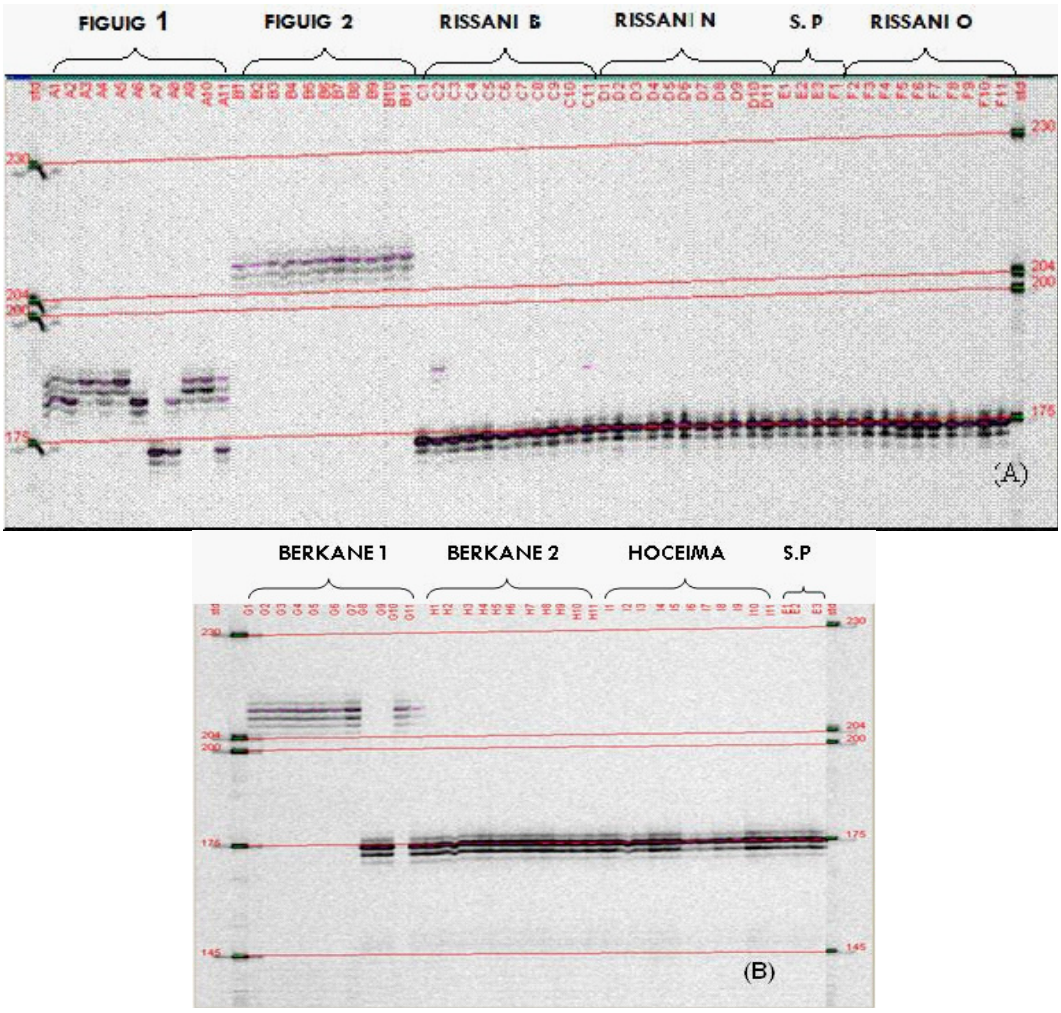

Figure 2. (A) and (B) Part of a gel obtained with the SSR primes Tom 236-237 and visualized in a LI-COR system. FIGUIG1, FIGUIG2, RISSANIB, RISSANIN, RISSANIO, BERKANE1, BERKANE2 and HOCEIMA correspond to tomato local cutivars. SP correspond to Saint Pierre commercial cultivar. Right and left extreme lanes represent 1kb DNA.

Table 3. Total number of polymorphic bands, seize range, number of alleles for 14 SSR markers used on 8 selected Moroccan tomato cultivars and one commercial variety.

Primer Name	Allelic Size Range (pb)	No. of Alleles	Polymorphic Band
SSR14	166-235	1	-
SSR22	208-214	2	2
SSR26	172-178	1	-
SSR63	206-248	4	4
SSR248	220-251	3	3
SSR578	290-299	1	-
Tom236-237	154-210	5	5
TOM184	163-206	2	2
TOM196-197	206-214	3	3
TOM210-211	216-222	3	3
TMS52	148-178	5	5
TMS56	102-126	3	3
TMS63	154-181	3	3
TMS65	288-298	4	4

Table 4. Pair-wise genetic distances from 14 SSR markers of eight tomato cultivars and one French commercial cultivar Saint Pierre.

	FIGUIG1	FIGUIG2	RISSANIB	RISSANIN	RISSANIO	BERKANE1	BERKANE2	HOCEIMA	Saint Pierre
FIGUIG1	1								
FIGUIG2	0,512	1							
RISSANIB	0,857	0,605	1						
RISSANIN	0,723	0,588	0,766	1					
RISSANIO	0,75	0,629	0,792	0,923	1				
BERKANE1	0,638	0,588	0,681	0,579	0,615	1			
BERKANE2	0,653	0,5	0,816	0,7	0,683	0,6	1		
HOCEIMA	0,741	0,634	0,852	0,622	0,652	0,8	0,723	1	
Saint Pierre	0,651	0,6	0,698	0,882	0,857	0,588	0,667	0,585	1

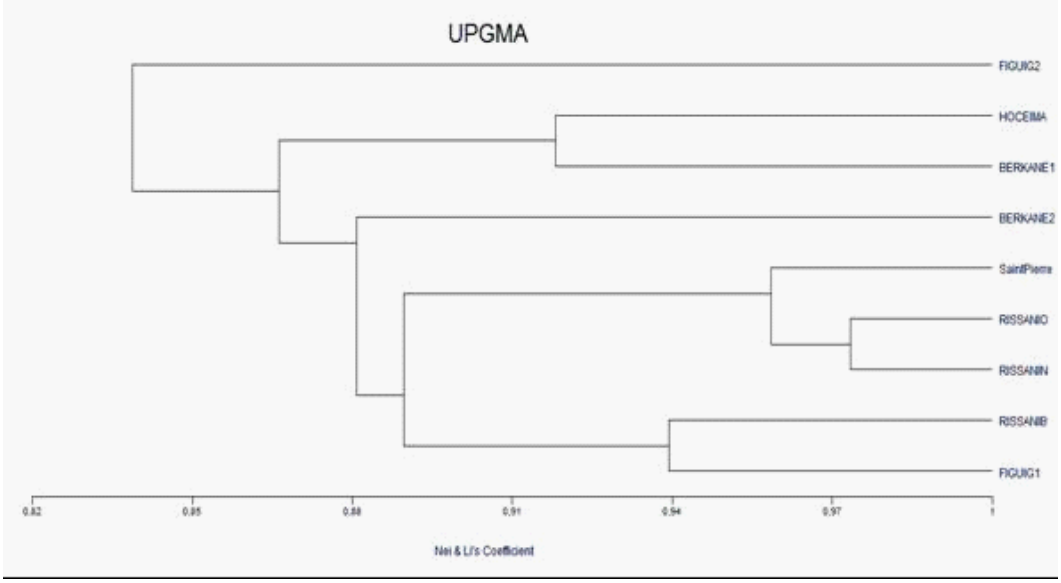

Figure 3. Dendrogram constructed from RAPD and SSRs data showing relationship among 8 of local Moroccan tomato cultivar and one French commercial tomato based on Nei and Li (1979) distance and the unweighted pairgroup arithmetic method (UPGMA).

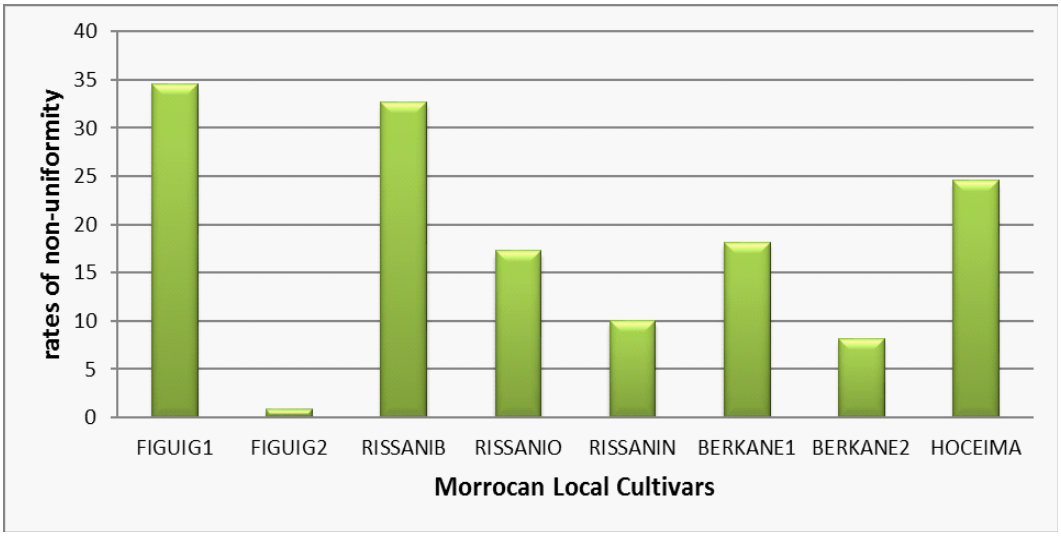

Figure 4. Rates of non-uniformity for Moroccan tomato cultivars tested.

and the fourth group includes only FIGUIG2. The latest genotype is clearly differentiated from the rest and could be used in tomato breeding program with specified objectives.

Simple Sequence Repeats Markers

Simple Sequence Repeats (SSR) or microsatellite markers have been successfully used to discriminate varieties which are morphologically similar and genetically close (Bredmeijer et al., 2002; He et al., 2003; Frary et al., 2005; Sarıkamıs et al., 2006, 2010). SSR markers may be adequate because of their high polymorphism, reproducibility, genetic co-dominance, easy detection, and multiallelic variation (Ruiz et al., 2005). Several studies have demonstrated the usefulness of SSR in cultivar identification (Bredmeijer et al., 1998; He et al., 2003). In this investigation, fourteen microsatellite markers were selected from the published data or on the website of Solanaceae Genomics Network (Table 1) and used to screen genetic diversity and genetic relationships among nine tomato cultivars. Three primers (21%) like SSR 14, SSR578 and SSR26 showed monomorphic profiles among all the screened tomato batches with only one amplified band (Table 3). In other studies, this percentage is much higher 49% as reported by Todorovska et al. (2014) and 25% by El-Awady et al. (2012). The other markers generated a polymorphic banding profile. The number of alleles amplified assessed from 2 alleles with primers Tom 184 and SSR 22 to 5 alleles with markers Tom 236-237 and TMS 52 (Figure 2) with a mean of 2.85 allele per locus. The scorable fragment sizes ranged from approximately 102 pb to 299 bp. Limited allelic variation was also observed in a study of eight tomato varieties and lines with an average of 3 alleles per locus after testing 160 SSR loci (Todorovska et al., 2014). El-Awady et al. (2012) showed also a low level of genetic diversity with an average of 2.1 alleles per locus by using 20 SSR markers in ten tested tomato cultivars.

To evaluate genetic relationship within the tomato lots, the data scored from the 14 SSR primers were analyzed on the basis of Nei & Li's Coefficient (1979). The relationship between tomato germplasm collected from different area in Morocco is illustrated by the data in Table 4. The genetic similarity estimated according to SSR data was scaled between 0.5 and 0.923 suggesting the potential of SSR markers in discriminating among plants compared to RAPD markers. In other studies this coefficient is much higher. For example, Archak et al. (2002) founded a similarity coefficients ranging between 0.610 - 0.976, Fanjuan et al. (2010) showed a genetic similarity varying between 0.72 - 1, Singh et al. (2014) reported a gene diversity from 0.65 to 0.97 underlying a weak genetic diversity in the tomato cultivars collection. The highest similarity value of 0.923 was shown between RISSANIN and RISSANIO while the lowest value 0.5 was observed between FIGUIG2 and BERKANE2. FIGUIG1 lot is distantly related to FIGUIG2 (49%) and relatively closed to RISSANIB (85%). HOCEIMA is close to BERKANE1 (80%) despite their diverse sources. RISSANIO and RISSANIB are distantly related to the certified control Saint Pierre 12% and 14% respectively suggesting an admixture of the control with those cultivated lots through hybridisation. The other lots are distantly related 30 to 42% to the control indicating a reduced germplasm diversity among the lot.

The distance matrix based on RAPDs and SSRs data was combined and used to construct a dendrogram (Figure 3). The dendrogram obtained can be divided into four main clusters, one contains only FIGUIG2 which formed a separate and more distant cluster. The second main cluster contains two cultivars each one is presented in one branch HOCEIMA and BERKANE1. The Third one includes only BERKANE1. The fourth cluster is divided into two sub-clusters, in one is branched Saint Pierre, RISSANIO and RISSANIN cultivars and the second one contains RISSANIB and FIGUIG1. Traditional tomato cultivars RISSANIO, RISSANIN on the one hand, RISSANIB and FIGUIG1 on the other hand are closely related to each other and seems to have common origin. This could be explained by the geographical situation of these region, RISSANI and FIGUIG are both situated in the south of Morocco and probably the farmers shared the same basis of the seeds.

The dendrogram showed the average of at least 5 grouping DNA samples per genotype. By calculating the rate of non-uniformity (Figure 4) we found considerable heterogeneity within the traditional cultivars. The rate of non-uniformity varies between the largest percentage (35%) in FIGUIG1 and the lowest (1%) in FIGUIG2. The level of heterogeneity found in the most traditional tomato cultivars could be characterized by a higher level of heterozigosity in some loci. On the other hand, FIGUIG2 possess a very high percentage of uniformity (99%) indicating that is a homogeneous traditional cultivar with high purity translating 100% homozygosity. This information could be efficiently used to establish a property rights and a germplasm conservation.

Conclusion

SSR markers used in this investigation were more suitable in the eight Moroccan local tomato cultivars as RAPD system. SSRs marker are better identification of tomato genotyping because they are codominant (Korir et al., 2014) while RAPD highlight only the dominant alleles. Except for FIGUIG2 cultivar, the most genotype studied are closely related despite their geographic sources.

Only the local FIGUIG2 cultivar shows the high degree of polymorphism since it was characterized with the highest number of unique bands (4) with RAPD markers and (5) with SSR markers in comparison with the other genotype. Furthermore, this lot hold the very high percentage of uniformity (99%) indicating that is a homogeneous traditional cultivar with high purity compared to the rest of tomato lots which are probably an admixture of commercial tomato. FIGUIG2 cultivar is a farmer-selected and adapted in area of local subsistence with semi-dry to dry climate and has low but stable annually yield. The polymorphism recorded in FIGUIG2 cultivar can be exploited in the management of genetic resources collection in Morocco and the establishment of property rights and protection. This genotype can be conserved and used in breeding programs and could offer gene combination to ensure adaptability and reproducibility in dried climate since genetic resources including landraces and wild relatives of crop species play an important role in breeding

programs (McCouch et al., 2013). In addition, A more accession survey will be necessary to evaluate a range of Moroccan tomato germplasm with more informative marker system and to establish a core collection in the gene banks that enabled the creation of a national plant genetic useful in breeding programs

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